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Sustained Activation of p38 Mitogen-Activated Protein Kinase and c-Jun N-Terminal Kinase Pathways by Hepatitis B Virus X Protein Mediates Apoptosis via Induction of Fas/FasL and Tumor Necrosis Factor (TNF) Receptor 1/TNF-α Expression

Wen-Horng Wang, Gérald Grégori, Ronald L. Hullinger, and Ourania M. Andrisani*

Department of Basic Medical Sciences, Purdue University, West Lafayette, Indiana

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Activation of the cellular stress pathways (c-Jun N-terminal kinase [JNK] and p38 mitogen-activated protein [MAP] kinase) is linked to apoptosis. However, whether both pathways are required for apoptosis remains unresolved. Hepatitis B virus X protein (pX) activates p38 MAP kinase and JNK pathways and, in response to weak apoptotic signals, sensitizes hepatocytes to apoptosis. Employing hepatocyte cell lines expressing pX, which was regulated by tetracycline, we investigated the mechanism of apoptosis by p38 MAP kinase and JNK pathway activation. Inhibition of the p38 MAP kinase pathway rescues by 80% the initiation of pX-mediated apoptosis, whereas subsequent apoptotic events involve both pathways. pX-mediated activation of p38 MAP kinase and JNK pathways is sustained, inducing the transcription of the death receptor family genes encoding Fas/FasL and tumor necrosis factor receptor 1 (TNFR1)/TNF-α and the p53-regulated Bax and Noxa genes. The pX-dependent expression of Fas/FasL and TNFR1/TNF-α mediates caspase 8 activation, resulting in Bid cleavage. In turn, activated Bid, acting with pX-induced Bax and Noxa, mediates the mitochondrial release of cytochrome *c*, resulting in the activation of caspase 9 and apoptosis. Combined antibody neutralization of FasL and TNF-α reduces by 70% the initiation of pX-mediated apoptosis. These results support the importance of the pX-dependent activation of both the p38 MAP kinase and JNK pathways in pX-mediated apoptosis and suggest that this mechanism of apoptosis occurs in vivo in response to weak apoptotic signals.

Apoptosis is a physiologically regulated process of programmed cell death involved in embryonic development and in the maintenance of homeostasis (33, 58). The dysregulation of apoptosis results in disease, e.g., cancer and autoimmune and neurodegenerative disorders (27, 75, 88). Apoptosis is also the basis for therapies designed to target cancerous cells and limit cytotoxicity that results from drug treatment (42). Thus, the molecular mechanisms and signaling pathways regulating apoptosis are of great significance.

The caspases mediating apoptosis include initiator caspases 8 and 9 and effector caspase 3 (25). Apoptosis occurs via two pathways: the extrinsic (death receptor) pathway, initiated by activation of members of the death receptor superfamily (Fas and tumor necrosis factor receptor 1 [TNFR1]), leading to caspase 8 activation (61), and the intrinsic (mitochondrial) pathway, resulting in the mitochondrial release of cytochrome *c* and caspase 9 activation (80). These two pathways converge upon the activation of caspase 3 (33). Mitochondrial involvement in apoptosis is determined by the balance of antiapoptotic (Bcl-2 and Bcl-X_L) and proapoptotic (Bax, Bad, Bid, and Noxa) Bcl-2 family members (46). Importantly, the extrinsic and intrinsic pathways are linked via the function of the protein Bid (80, 81).

Apoptosis is regulated by the p38 mitogen-activated protein (MAP) kinase/c-Jun N-terminal kinase (JNK) cellular stress pathways (47, 76). These pathways, members of the mitogenic family of signaling cascades (39), also mediate proliferation and differentiation (20, 26, 42, 62, 64, 65). Evidence in support of JNK and p38 MAP kinase pathways in regulating apoptosis is derived from studies employing treatments simulating cellular stress. These stresses include growth factor withdrawal (21, 85), the presence of proinflammatory cytokines (8, 56, 67) and drugs (17, 29, 57, 63), UV radiation (79), and overexpression of constitutively active effectors, e.g., MEKK1 (40), ASK-1 (31, 41), and JNK1 (51). However, despite reports of a role for the JNK and p38 MAP kinase in apoptosis and the demonstration that ASK-1 is upstream of both JNK and p38 MAP kinase pathways (37, 78), whether both pathways are necessary for apoptosis remains unresolved. For example, although both the p38 MAP kinase and JNK pathways are activated upon exposure to UV radiation (34), only the JNK pathway mediates UV-induced apoptosis in primary mouse fibroblasts (79). With other apoptotic treatments, e.g., by overexpression of constitutively active ASK-1 (31, 41), activation of the JNK pathway is the dominant event in mediating apoptosis, although ASK-1 is known to activate both the p38 MAP kinase and JNK pathways (37, 78). Thus, despite the demonstrated role of the JNK pathway in apoptosis under robust apoptotic conditions, it is not yet

* Corresponding author. Mailing address: Department of Basic Medical Sciences, Purdue University, West Lafayette, IN 47907-1246. Phone: (765) 494-8131. Fax: (765) 494-0781. E-mail: andrisao@purdue.edu.

understood whether activation of both the p38 MAP kinase and JNK pathways is required for apoptosis occurring under physiologically relevant conditions. The present study was undertaken to understand the role of the concurrent activation of the p38 MAP kinase and JNK stress pathways in apoptosis.

We employed a well-characterized cellular model (49, 71–73) in which sustained activation of the JNK and p38 MAP kinase pathways is mediated by tetracycline-regulated expression of the hepatitis B virus (HBV) X protein (pX). pX is required for the viral life cycle (90) and is implicated in the hepatocarcinogenesis of chronic HBV patients (11). In addition, pX sensitizes hepatocytes to apoptosis in response to weak apoptotic stimuli, such as subapoptotic concentrations of transforming growth factor β (TGF-β) or TNF-α (43, 69) by an unknown mechanism. pX induces the activation of mitogenic Ras-Raf-MAP kinase (5, 23, 73), JNK (6, 73), and p38 MAP kinase (72) pathways via calcium-regulated activation of c-Src (9, 44). Importantly, earlier studies (72, 73) employing pX-expressing hepatocyte cell lines demonstrated that tetracycline-regulated pX differentially activates the Ras-Raf-MAP kinase, JNK, and p38 MAP kinase pathways in differentiated versus less differentiated hepatocytes. In differentiated hepatocytes, modeled by the AML12 3pX-1 cell line, conditional pX expression activates the Ras-Raf-MAP kinase pathway in a sustained manner (72). By contrast, in the less differentiated AML12 4pX-1 cell line, conditional pX expression mediates the sustained activation of the JNK (73) and p38 (72) pathways.

Since pX sensitizes cells to apoptosis (43, 69) and the JNK and p38 MAP kinase pathways are linked to apoptosis (79), the aim of this study using the conditional pX-expressing 4pX-1 cell line was to define the role of the activation of both stress pathways (JNK and p38 MAP kinase) in apoptosis and the mechanism of pX-mediated hepatocyte apoptosis. Considering that apoptosis is a physiologically regulated process, the significance of our cellular model of HBV pX-mediated apoptosis is that it models apoptosis in response to a weak, physiologically relevant apoptotic stimulus.

In this study, we demonstrate for the first time how the activation of both the p38 MAP kinase and JNK pathways mediates apoptosis in response to the weak apoptotic stimulus of HBV pX, and we provide a mechanistic understanding of how HBV pX mediates apoptosis in hepatocytes. Considering the fact that the suppression of apoptosis often leads to the development of cancer (27), this study identifies key regulatory steps that may be deregulated in HBV-mediated hepatocarcinogenesis. Importantly, reduced p38 MAP kinase activity is detected in human liver tumor samples relative to levels in nontumorous controls (38), and the loss of p38 MAP kinase

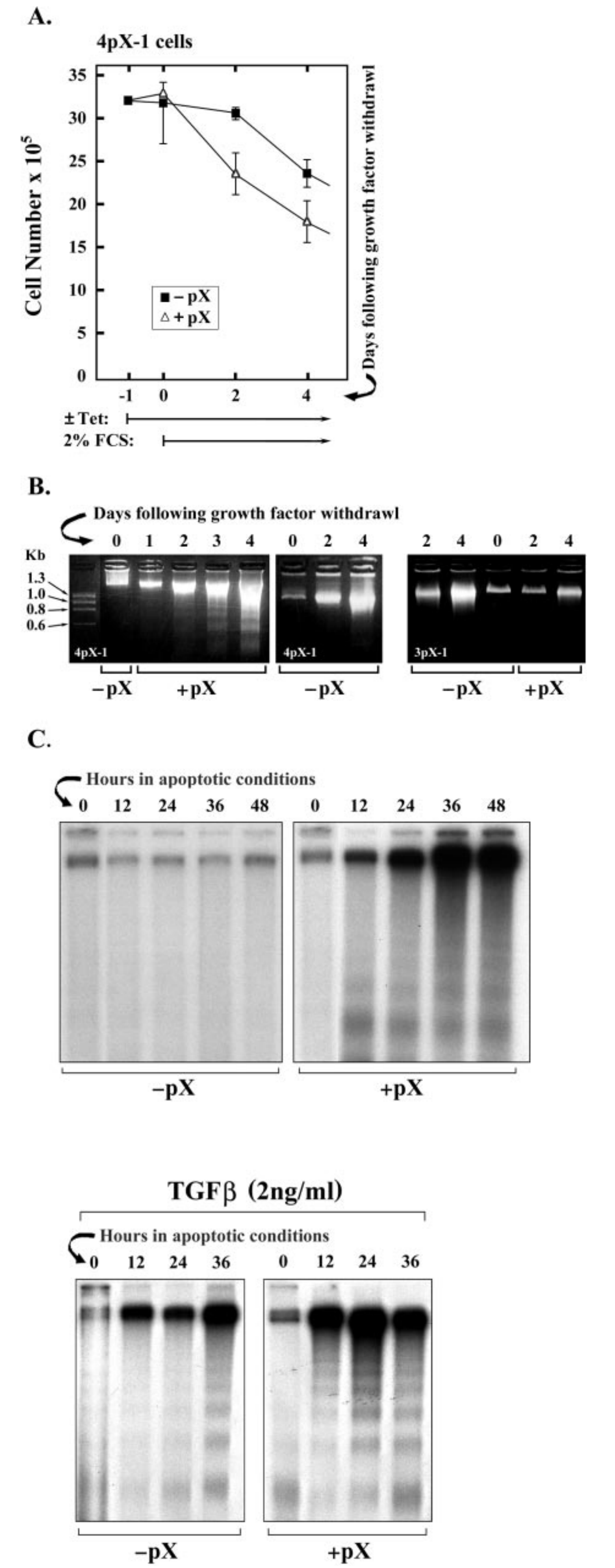


FIG. 1. Growth conditions for detecting pX-mediated apoptosis in a 4pX-1 cell line expressing pX, which is regulated by tetracycline. (A) Confluent 4pX-1 cultures were grown with and without 5 μg of tetracycline (Tet) per ml starting at day −1 and were switched to 2% FCS at day 0. Cell numbers were measured from triplicate cultures. Results are from three independent experiments ($P < 0.005$). (B) DNA fragmentation assays of 4pX-1 or 3pX-1 DNA isolated from confluent cultures at the indicated times following growth factor withdrawal, with (+) or without (−) pX expression. (C) Radioactive DNA fragmentation assays (71) of DNA from 4pX-1 cultures grown under conditions described for panel A. "Hours in apoptotic conditions" indicates growth in 2% FCS with (+) pX or without (−) pX expression.

activation is associated with increased tumorigenesis in mutant cells (10).

MATERIALS AND METHODS

Cell culture. Cell lines 3pX-1 and 4pX-1 derived from AML12 cells were propagated as described previously (71, 73) in media containing 5 μg of tetracycline per ml to turn off pX expression. On day −1 (Fig. 1A), confluent 4pX-1 cells were treated or not treated with 5 μg of tetracycline per ml in media containing 10% fetal calf serum (FCS), and on day 0 (Fig. 1A), the growth media were switched to 2% FCS with or without 5 μg of tetracycline per ml. Time course apoptotic assays were performed 12 to 48 h following incubation in 2% FCS.

Inhibitors used were SB 202190 (5 μM; CalBiochem), SP 600125 (5 μM; Tocris), the p53 transcriptional inhibitor (20 μM; BioMoL), Z-IETD-fluoromethyl ketone (FMK) (1 μM; Biovision), Z-LEHD-FMK (0.7 μM; Biovision), Z-DEVD-FMK (1 μM; Biovision), carboxyfluorescein (FAM)-LETD-FMK (BIOCARTA), and FAM-LEHD-FMK (BIOCARTA).

Transient transfections of luciferase reporters were performed using FuGENE 6 (Roche) reagent as described previously (72, 73). DNA was added 8 h prior to serum withdrawal on day 0 (Fig. 1) and harvested by day 2. Assays were performed in triplicate, and results were quantified per microgram of protein extract.

Real-time quantitative PCR was performed as described previously (49), with 18S RNA as the internal control. The forward and reverse primers used were as follows: for Bax, 5′-TGGATAGCAATATGGAGC-3′ and 5′-AAGTAGAAGAGGGCAACC-3′; for Fas, 5′-CCAGTCGTGAAACCATACC-3′ and 5′-CTCATCTATCTTGCCCTCC-3′; for FasL 5′-ACTACCACCGCCATCACAACC-3′ and 5′-CAACCAGAGCCACCAGAACC-3′; for Noxa, 5′-GAGACAAAGTGTATTGCACG-3′ and 5′-GTCCTTCAAGTCTGCTGG-3′; for TNFR1, 5′-GAGAAAGTGAGTGCGTCC-3′ and 5′-CAAAGACCTAGCAAGATAACC-3′; and for TNF-α, 5′-CACCATGAGCACAGAAAGC-3′ and 5′-CAAGCAGGAATGAGAAGAGG-3′. DNA fragmentation assays were performed as described previously (77). Briefly, 10^6 4pX-1 cells grown under apoptotic conditions were resuspended in 400 μl of buffer containing 10 mM Tris (pH 7.4), 20 mM EDTA, and 0.5% Triton X-100 and incubated on ice for 10 min, followed by the addition of 0.1% sodium dodecyl sulfate. Genomic DNA was isolated and radiolabeled with [^{32}P]dCTP using DNA polymerase I (New England Biolabs) at 4°C for 2 h. Analysis was by 2% agarose gel electrophoresis and phosphorimager quantification. FasL Kay-10 antibody purchased from Pharmingen and TNF-α antibody clone MPG-XT3 from Upstate Biotechnology were used at 1 μg/ml versus control immunoglobulin G (2 μg/ml) in antibody neutralization experiments of apoptosis.

In vitro Cdc2 kinase assays were performed as described previously (49).

Western blot analyses were performed with cellular extract isolated from apoptotic 4pX-1 cell cultures with and without 5 μg of tetracycline per ml. Phospho-specific antibodies for p38 MAP kinase and JNK and antibodies for unmodified p38 MAP kinase and JNK were from Upstate Biotechnology. Antibodies for caspases 8 and 9 were purchased from Alexis, those for Bid were purchased from Cell Signaling Technology, and cytochrome *c* was purchased from BD Biosciences. Antibodies for p53 and phospho-p53 (Ser15) were from Cell Signaling. The mitochondrial fractionation kit was from BD Biosciences. Cellular extracts were prepared from apoptotic 4pX-1 cultures (10^7 cells) harvested in 200 μl of 1× sodium dodecyl sulfate sample loading buffer. Western blot analyses were performed with the Amersham enhanced-chemiluminescence reagent.

Flow cytometry. In situ labeling of active caspases in live 4pX-1 cells (10^5 cells) was performed for 1 h at 37°C with 5% CO_2 with a FAM-labeled peptide FMK caspase 8 or 9 inhibitor (BIORCARTA). The optimal concentration for each fluorogenic caspase inhibitor was determined by titration analyses. Following in situ labeling, cells were collected and washed twice (with buffer supplied by the manufacturer), resuspended in buffer containing 10 μg of propidium iodide per ml, and analyzed by flow cytometry with a Cytomics FC 500 flow cytometer (Beckman-Coulter) at a flow rate of <1,000 cells/s. Data were collected and stored in real time with RXP software (Beckman-Coulter). Analyses were performed with WinList 5.0 software (Verity Software House). Discrimination of apoptotic cells and determination of their abundance were performed on the green (525-nm-wavelength) as opposed to red (675-nm-wavelength) fluorescence cytogram.

Statistical analyses were performed with Scion software.

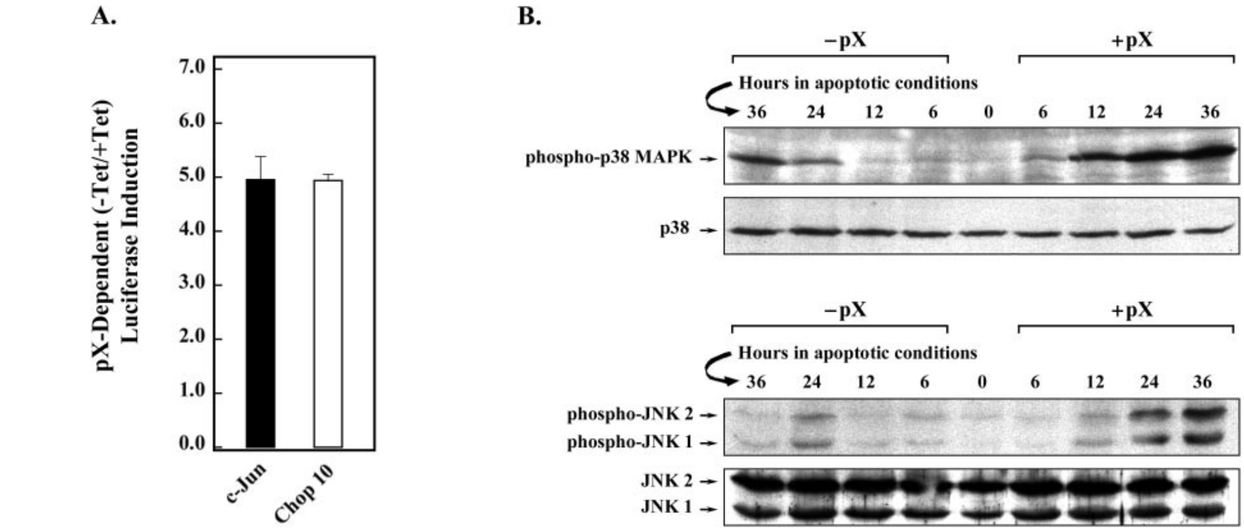
RESULTS

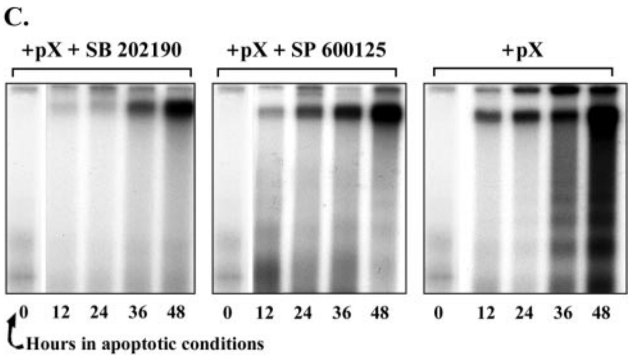
In 4pX-1 cells, a less differentiated hepatocyte cell line than 3pX-1 cells, pX mediates sustained JNK and p38 MAP kinase pathway activation (72, 73), whereas in the differentiated hepatocyte 3pX-1 cell line, pX mediates sustained activation of the Ras-Raf-MAP kinase pathway (73). Since the JNK, p38 MAP kinase pathways have been linked to apoptosis, we employed the tetracycline-regulated, pX-expressing 4pX-1 cell line (71) to determine the early signaling events involved in pX-mediated hepatocyte apoptosis.

To detect pX-mediated apoptosis (Fig. 1A), we employ confluent 4pX-1 cultures, with pX expression (by tetracycline removal) being initiated at day −1 in media containing 10% FCS, followed by incubation on day 0 in 2% FCS. Importantly, although these conditions resemble growth factor deprivation, they sustain pX synthesis (71–73). A small but reproducible increase in 4pX-1 cell number is observed following pX expression for 24 h in media containing 10% FCS, i.e., from day −1 to day 0. Continued incubation in 2% FCS, in the presence of pX starting at day 0, results in a statistically significant ($P <$ 0.005) decrease in cell number, relative to numbers in cultures grown without pX (Fig. 1A). Employing DNA fragmentation assays, we demonstrate that pX promotes increased DNA fragmentation, evident by day 2, under these growth conditions (Fig. 1B). Interestingly, expression of pX in the differentiated 3pX-1 cell line under the same apoptotic conditions does not induce DNA fragmentation (Fig. 1B). Since DNA fragmentation is a strong criterion of apoptosis, we conclude that under conditions of growth factor deprivation, pX sensitizes less differentiated hepatocytes to apoptosis.

By a radioactive DNA fragmentation assay (77), pX-dependent apoptosis is detectable as early as 12 h following growth factor withdrawal (Fig. 1C). Earlier studies have shown that pX increases the sensitivity of cells to apoptosis (69). Accordingly, we treated 4pX-1 cultures with low concentrations of the proapoptotic agents TGF-β, doxorubicin, and staurosporine. In agreement with the earlier observations (69), our results show that pX expression increases DNA fragmentation of 4pX-1 cells treated with a subapoptotic concentration of TGF-β (Fig. 1C), doxorubicin, and staurosporine (data not shown). Furthermore, similar analyses of other clonal isolates derived from the 4pX lineage, such as 4pX-2 and 4pX-3 cells (49, 71), demonstrated that pX had the same apoptotic effect.

The p38 MAP kinase pathway contributes to initiation of pX-mediated apoptosis. Earlier studies (72, 73) demonstrated that pX induces sustained activation of JNK and p38 MAP kinase pathways in 4pX-1 cells. Employing transient-transfection trans-reporter assays and apoptotic 4pX-1 cultures, we demonstrate a fivefold pX-dependent activation of p38 MAP kinase and JNK pathways (Fig. 2A) via the expression plasmids Ga14-Chop-10 and Ga14-c-Jun, respectively. The p38 MAP kinase and JNK pathways activate these fusion proteins by phosphorylation, which in turn promotes the expression of the pFR-luciferase reporter. Employing phospho-specific antibodies for the p38 MAP kinase and JNK enzymes, we determined the kinetics of activation of these pathways following the onset of pX-mediated apoptosis (Fig. 2B). Under apoptotic 4pX-1 conditions, pX-dependent activation of p38 MAP kinase occurs within 6 h, whereas JNK activation is delayed, occurring





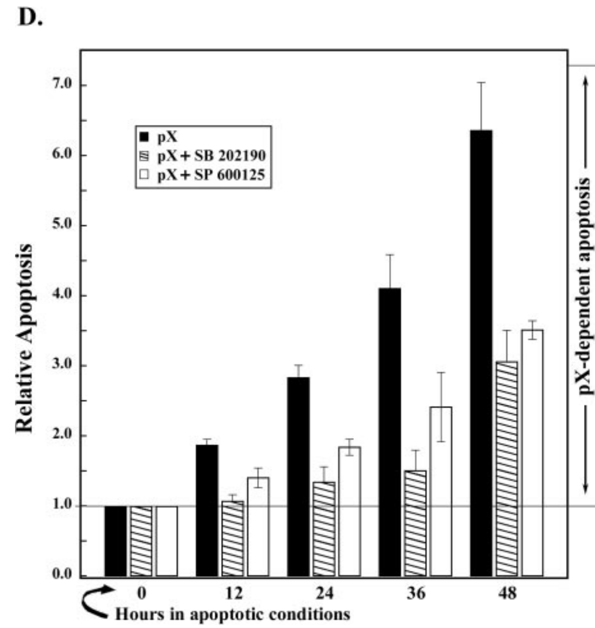


FIG. 2. Combined p38 MAP kinase and JNK activation in pX-mediated apoptosis. (A) Transient-transfection trans-reporter assays employing the pFR luciferase reporter (100 ng) cotransfected with the pFA-c-Jun (100 ng) or pFA-Chop-10 (100 ng) expression vectors (Stratagene) in apoptotic 4pX-1 cultures, which were grown in the presence or absence of 5 μg of tetracycline per ml. Results are from three independent assays performed in triplicate. (B) Western blot analyses of phospho-p38 MAP kinase and phospho-JNK using apoptotic 4pX-1 whole-cell extracts isolated at the indicated times as a function of pX expression. The results of a representative assay from three independent experiments are shown. (C) Radioactive DNA fragmentation assays of DNA isolated from apoptotic 4pX-1 cultures in the presence or absence of 5 μM SB 202190 or in the presence or absence of 5 μM SP 600125. (D) Quantification of results of radioactive DNA fragmentation assays performed with ImageQuant (version 5.0; Molecular Dynamics). Results are representative of three independent assays ($P < 0.01$).

by 12 h after the initiation of pX-dependent apoptosis. Interestingly, both pathways display sustained activation at 36 h, following the onset of pX-dependent apoptosis (Fig. 2B).

To determine the involvement of these pathways in pX-mediated apoptosis, apoptotic assays were performed as a function of the addition of known specific inhibitors of p38 MAP kinase and JNK (Fig. 2C and D). The effective concentration of these inhibitors, SB 202190 for the p38 MAP kinases α and β and SP 600125 for JNK1 and -2, was selected as 5 μM by titration assays (data not shown) in order to prevent cytotoxicity. Importantly, 5 μM is well in excess of the known 50% inhibitory concentration of each inhibitor but below the concentration known to cause nonspecific inhibition of MAP kinase enzymes (7, 19).

The effect of SB 202190 or SP 600125 was measured 12 to 48 h following the incubation of 4pX-1 cultures under apoptotic conditions in the presence of pX expression (Fig. 2C). Apoptotic 4pX-1 cultures grown without inhibitors in the presence of pX display a progressive increase in apoptosis, from twofold (12 h) to sixfold (48 h), relative to the zero time point. This apoptosis increase, following incubation of the cultures under low-concentration serum conditions, represents pX-mediated sensitization to apoptosis (Fig. 2D). By contrast, 4pX-1 cultures grown without pX under low-concentration serum conditions for 48 h demonstrate minimal apoptosis (Fig. 1). Inhibition of the p38 MAP kinase pathway by the addition of SB 202190 results in a dramatic (80%) rescue of the initial phases (12 to 36 h) of pX-mediated apoptosis. By contrast, at

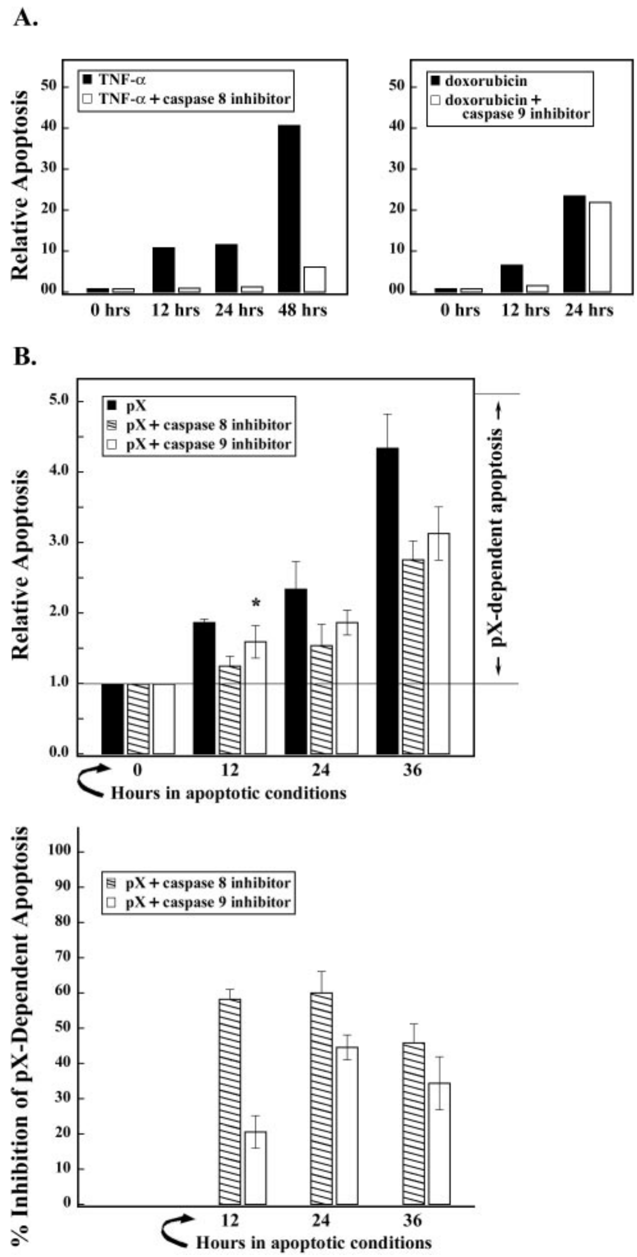


FIG. 3. Caspase 8 is the major effector of pX-mediated apoptosis. (A) 4pX-1 cells grown with 5 μg of tetracycline per ml and treated with TNF-α (20 ng/ml) or doxorubicin (1 μM) with (open bars) or without (filled bars) 1 μM Z-IETD-FMK (caspase 8 inhibitor) (left panel) or 1 μM Z-LEHD-FMK (caspase 9 inhibitor) (right panel). (B) Apoptotic 4pX-1 cultures expressing pX were grown without tetracycline, treated with 1 μM Z-IETD-FMK or 0.7 μM Z-LEHD-FMK, and analyzed by radioactive DNA fragmentation assays. Quantification was by ImageQuant (version 5.0; Molecular Dynamics). Percentages of inhibition of pX-dependent apoptosis are shown in the lower panel. Results are representative of three independent assays ($P < 0.05$). * indicates the absence of statistical significance.

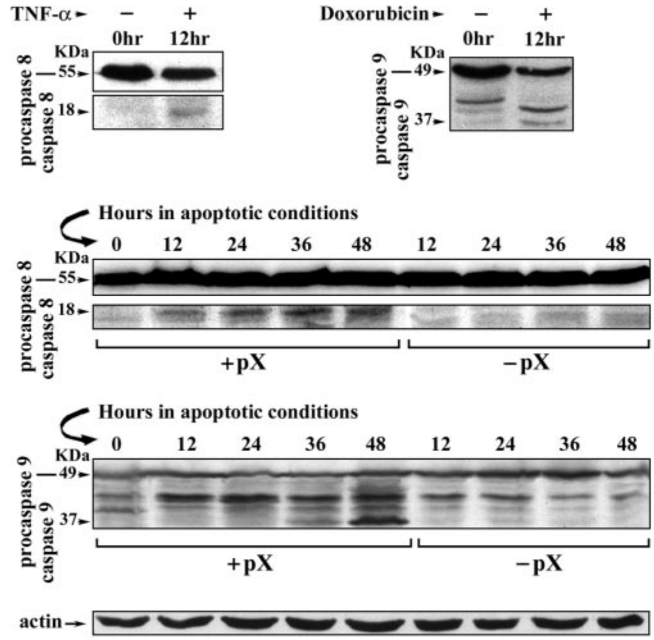


FIG. 4. Caspase 8 mediates the initiation of pX-mediated apoptosis. Shown are the kinetics of the pX-dependent activation of caspases 8 and 9 as determined by Western blot analyses of whole-cell extracts isolated from apoptotic 4pX-1 cultures grown in the presence (+) or absence (−) of pX. Pro-caspases 8 and 9 and their corresponding active forms are indicated by arrows. The same whole-cell extracts were analyzed for caspase 8 and 9 activation by employing actin as the loading control. Controls included 4pX-1 cells grown in the presence of 5 μg of tetracycline per ml and treated with TNF-α (20 ng/ml) or doxorubicin (2 μM) for the indicated times. The results of a representative assay from three independent experiments are shown.

48 h, both the p38 MAP kinase and JNK pathways contribute equally to pX-dependent apoptosis. These results are in agreement with the kinetics of the pX-dependent, sustained activation of the p38 MAP kinase and JNK enzymes shown in Fig. 2B.

Caspase 8 is the initiator caspase of pX-mediated apoptosis. To elucidate further the mechanism of pX-mediated apoptosis, we determined the contribution of each initiator caspase, employing cell-permeable peptide inhibitors for caspase 8 (Z-IETD-FMK) and caspase 9 (Z-LEHD-FMK).

Inhibitor activity was confirmed in apoptosis assays (Fig. 3A) carried out with control (with tetracycline) 4pX-1 cultures by treatment with TNF-α (20 ng/ml) or doxorubicin (1 μM), agents known to activate caspases 8 and 9, respectively. Addition of 1 μM Z-IETD-FMK (caspase 8 inhibitor) inhibits TNF-α-mediated apoptosis by approximately 80 to 90% in the 12- to 48-h interval (Fig. 3A). Similarly, 1 μM Z-LEHD-FMK (caspase 9 inhibitor) inhibits doxorubicin-mediated apoptosis by 75% within 12 h (Fig. 3A). However, at 24 h, doxorubicin mediates caspase 9-independent apoptosis, probably due to irreversible destruction of mitochondria.

In our study of pX-mediated apoptosis, the optimal concentration of caspase 8 and 9 inhibitors was determined by titration assays following conditions of growth factor deprivation (data not shown). Employing these optimal conditions, sensitization to pX-dependent apoptosis was assessed as a function of caspase 8 or 9 inhibition. The caspase 8 inhibitor (Z-IETD-FMK) reduces sensitization to pX-mediated apoptosis by 60% at the 12- to 24-h interval, whereas the caspase 9 inhibitor (Z-LEHD-FMK) results in approximately 20% apoptosis reduction at 12 h, reaching approximately a 40% reduction at the

24- to 36-h interval (Fig. 3B). Similar assays performed with the caspase 3 inhibitor (Z-DEVD-FMK) demonstrate a 70% inhibition (data not shown).

To further confirm these observations, we performed Western blot analysis and monitored the kinetics of the appearance of the active forms of caspases 8 and 9 during the 12- to 48-h interval (Fig. 4). Active caspase 8 is detected at 12 h following the initiation of pX-mediated apoptosis reaching the maximal level by 48 h. By contrast, active caspase 9 is detected at 36 h following the onset of apoptosis (Fig. 4).

To directly monitor the activation of caspases 8 and 9, we employed specific fluorochrome-conjugated inhibitors. These caspase substrates are cell-permeable carboxyfluorescein-labeled FMK-peptides that bind covalently to the active caspase. The fluorogenic substrate fluoresces upon cleavage by the caspase, enabling detection of the active caspase in situ as cells undergo apoptosis. Figure 5A demonstrates the specificity of these fluorogenic inhibitors in detecting activated caspases in situ. Employing these reagents, we detect activation of caspase 8 (Fig. 5B) within 12 h after the initiation of pX-mediated apoptosis and a progressive and pronounced increase in caspase 8 activation (both intensity and cell number) occurring in the 24- to 36-h interval. In parallel assays, caspase 9 activation (Fig. 5C) is delayed, displaying detectable activation starting at 24 h and a pronounced increase 36 h following the onset of pX-mediated apoptosis. Quantification by flow cytometry of the caspase 8- or 9-positive cells (Fig. 5D) demonstrates that caspase 8 is the initiator caspase in pX-dependent apoptosis. pX-dependent caspase 8 activation displays a progressive increase, initiated during the early phases (6 to 24 h) of pX-dependent apoptosis, followed by the delayed (24 to 36 h) activation of caspase 9. In addition, employing this flow cytometric assay in conjunction with the p38 MAP kinase inhibitor (SB 202190) or JNK inhibitor (SP 600125), we have determined that at 24 h the p38 MAP kinase pathway contributes approximately 50% to caspase 8 activation versus a 25% contribution by the JNK pathway (data not shown). These results agree with the early and major involvement of the p38 MAP kinase pathway in pX-dependent apoptosis (Fig. 2D).

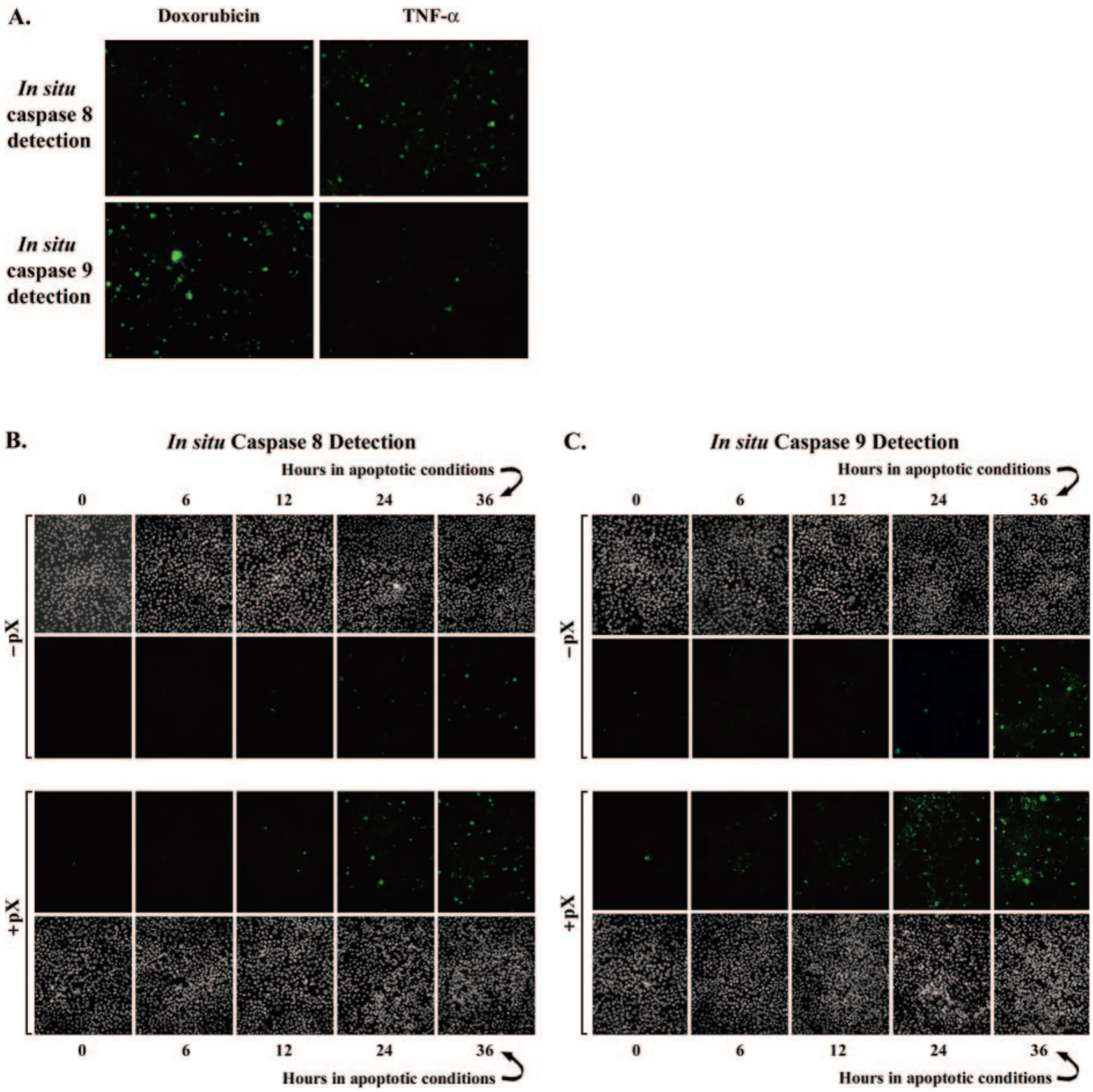
Kinetics of Bid activation and mitochondrial involvement. A target of active caspase 8 is Bid, a 22-kDa BH3-only protein (33). Cleavage of Bid by caspase 8 generates the death-promoting p15 t-Bid, which targets mitochondria (53, 55). Our results show that caspase 8 is the initiator caspase of pX-mediated apoptosis (Fig. 3 and 5D) and that its activation occurs within 12 h following the onset of pX-dependent apoptosis (Fig. 3 to 5). Accordingly, we monitored by Western blotting the kinetics of Bid cleavage and investigated the role of the p38 MAP kinase and JNK pathways in Bid activation (Fig. 6A). The cleavage of Bid is detectable by 12 h following the onset of pX-dependent apoptosis, reaching a maximum at 48 h. This cleavage of Bid is significantly inhibited by the addition of the p38 MAP kinase inhibitor (SB 202190), whereas the JNK inhibitor (SP 600125) has a minimal effect (Fig. 6A).

To further confirm these observations, we investigated the kinetics of mitochondrial cytochrome *c* release into the cytosolic fraction (Fig. 6B). Following the onset of pX-dependent apoptosis, cytosolic cytochrome *c* is detected starting at 12 h and reaches a maximum by 48 h. Treatment with either SB 202190 or SP 600125 blocked cytochrome *c* release (Fig. 6B), indicating involvement of both pathways in this process.

Mechanism of p38 MAP kinase and JNK pathway involvement in pX-mediated apoptosis. Earlier studies (13, 82) demonstrated a link between p38 MAP kinase activation induced by UV irradiation and inactivation of Cdc25. In turn, Cdc25 regulates Cdc2 activation required for progression to mitosis. Previous studies also demonstrated that pX-dependent p38 MAP kinase pathway activation arrests 4pX-1 cells at the G_2/M checkpoint via Cdc25 inactivation (72). Employing in vitro Cdc2 immunocomplex kinase assays, we demonstrate minimal Cdc2 activation in apoptotic 4pX-1 cells expressing pX at the 12- to 24-h interval (Fig. 7). Treatment of apoptotic 4pX-1 cultures with SB 202190, inhibiting the p38 MAP kinase pathway, restores pX-dependent Cdc2 activation (Fig. 7). These observations, together with the results shown in Fig. 2, indicate that one aspect of p38 MAP kinase pathway-mediated apoptosis in 4pX-1 cells is activation of the G_2/M checkpoint.

p38 MAP kinase and JNK pathways transcriptionally induce endogenous 4pX-1 proapoptotic genes. To understand further how pX-dependent activation of the p38 MAP kinase and JNK pathways mediates apoptosis via activation of caspase 8, we investigated whether these stress pathways induce the expression of genes activating the extrinsic, death receptor pathway. Employing luciferase reporter constructs containing the Fas and FasL gene promoters (Fig. 8A), we demonstrate pX-dependent transcriptional induction from these promoters in apoptotic 4pX-1 cultures. Interestingly, pX-dependent induction of Fas- or FasL-driven transcription does not occur in the differentiated 3pX-1 cell line (data not shown), a cell line that does not display pX-dependent apoptosis (Fig. 1B). In addition, the Bax-luciferase reporter also displays pX-dependent induction. Bax is proapoptotic and participates in the mitochondrial release of cytochrome *c* and the activation of caspase 9. Bax gene transcription is p53 dependent, and addition of its transcriptional inhibitor pFTα blocks Bax reporter expression as well as pX-dependent apoptosis (data not shown), in agreement with previous reports (15). Importantly, the pX-dependent induction of the Bax-luciferase reporter is not observed in differentiated 3pX-1 cells (reference 49 and data not shown), which are insensitive to pX-dependent apoptosis (Fig. 1B).

To confirm the transient-transfection results (Fig. 8A), we investigated by real-time PCR (49) the pX-dependent induction of two classes of endogenous proapoptotic 4pX-1 genes. Those involved in the activation of the extrinsic apoptotic pathway include the genes for Fas/FasL and TNFR1/TNF-α and the p53-regulated Bax and Noxa genes, which participate in the intrinsic, mitochondrial apoptotic pathway. Real-time-PCR analyses demonstrate that both classes of endogenous proapoptotic 4pX-1 genes display pX-dependent transcriptional induction at the 12- to 24-h interval of pX-dependent apoptosis (Fig. 8B). We also examined the effect of inhibition of the p38 MAP kinase and JNK pathways on the expression of these endogenous proapoptotic genes at 12 and 24 h after the onset of apoptosis. We observed the involvement of both pathways in the transcriptional induction of the Fas/FasL and TNFR1/TNF-α genes (Fig. 8C). Similar results are also observed with the transcriptional induction of Bax and Noxa (data not shown). Further, to confirm that the induction of the p53-



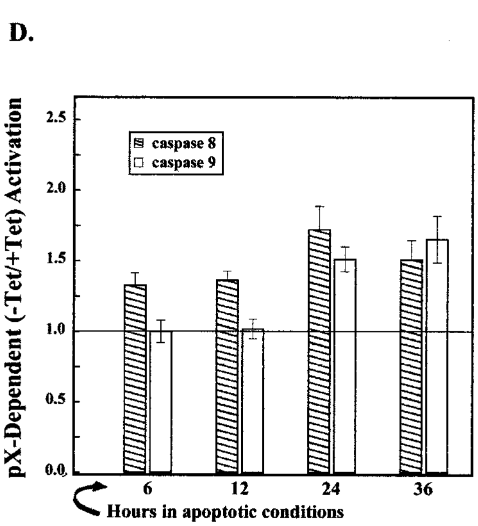


FIG. 5. In situ detection of activated caspases in apoptotic 4pX-1 cells. (A) 4pX-1 cultures (with tetracycline) treated with doxorubicin (1 μM) or TNF-α (20 ng/ml) were incubated with the recommended amount (BIOCARTA) of the carboxyfluorescein substrate FAM-LETD-FMK (caspase 8) or FAM-LEHD-FMK (caspase 9). Cells were washed with PBS and visualized by fluorescence microscopy. (B and C) In situ detection of active caspase 8 (B) and caspase 9 (C) in apoptotic 4pX-1 cultures grown in the presence (+) or absence (−) of pX and incubated with FAM-LETD-FMK or FAM-LEHD-FMK, respectively. Upper (−pX) and lower (+pX) panels show Hoechst staining of nuclei at a 20× magnification. (D) Flow cytometric analyses of apoptotic 4pX-1 cell cultures harvested at the indicated times following the onset of pX-mediated apoptosis and stained with the fluorogenic FAM-LETD-FMK caspase 8 inhibitor or the FAM-LEHD-FMK caspase 9 inhibitor and propidium iodide. Quantifications of cells are from three independent experiments ($P < 0.001$).

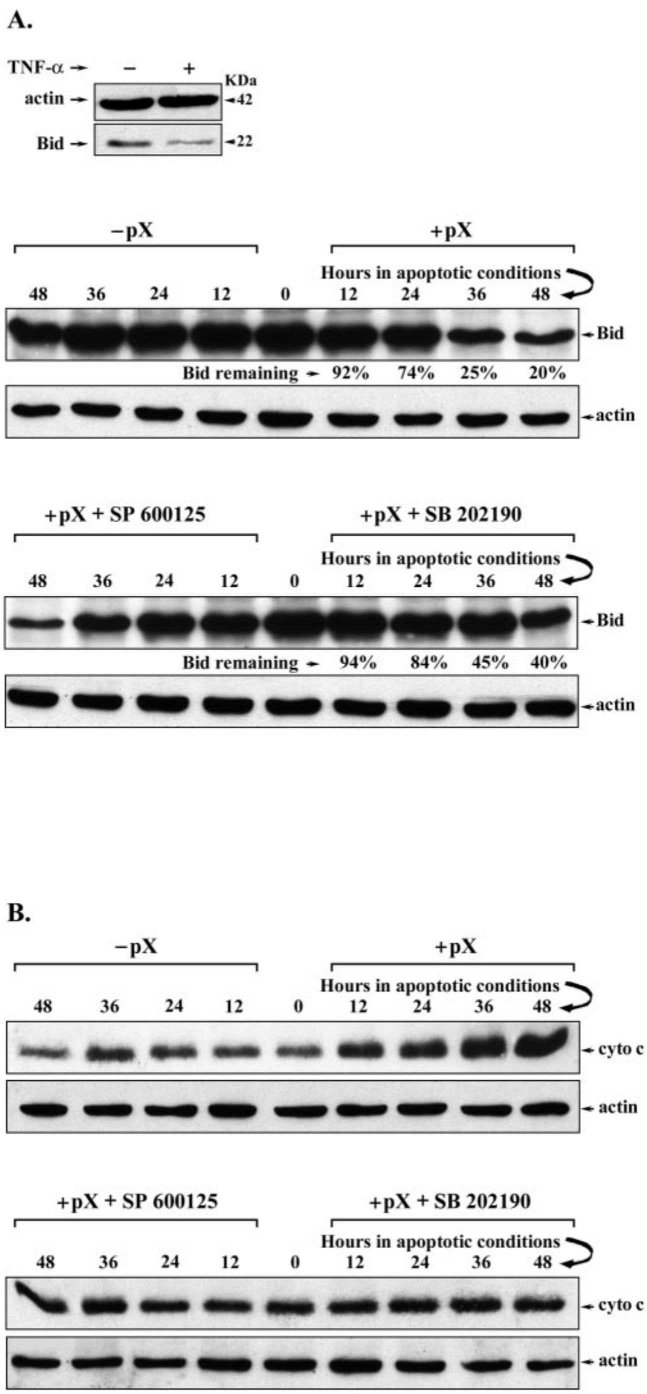


FIG. 6. Kinetics of Bid cleavage and cytochrome *c* release in pX-mediated apoptosis. (A and B) Western blot analysis monitoring Bid cleavage (A) and cytosolic cytochrome *c* (cyto c) appearance (B) in apoptotic 4pX-1 cultures grown with (+) or without (−) pX in the presence (+) or absence (−) of SB 202190 (5 μM) or SP 600125 (5 μM), as indicated. The Western blot of actin is the loading control. In panel A, Western blot analysis of Bid cleavage was with whole-cell extracts isolated from 4pX-1 cultures (with tetracycline) and treated with TNF-α (20 ng/ml) for 12 h. The results of a representative experiment from three independent assays are shown.

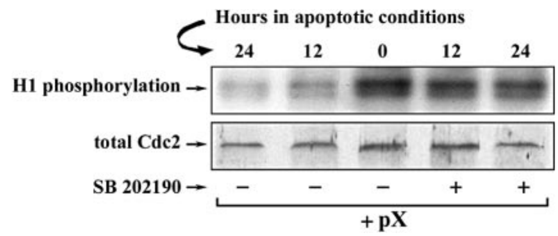


FIG. 7. p38 MAP kinase prevents Cdc2 activation in apoptotic 4pX-1 cultures. Shown are the results of Cdc2 immunocomplex kinase assays employing histone H1 as the substrate and whole-cell extracts, which were isolated at the indicated times from apoptotic 4pX-1 cultures grown in the presence of pX and with (+) or without (−) SB 202190 (5 μM). Total Cdc2 protein was monitored by Western blotting with Cdc2 antibody. The results of a representative experiment from three independent assays are shown.

regulated Bax and Noxa genes coincides with increased p53 protein levels, we performed Western blot assays for the p53 protein (Fig. 8D). pX-dependent p53 stabilization is observed within 1 h following incubation under apoptotic conditions (Fig. 8D).

The mechanism of p53 stability is complex, involving multiple phosphorylations (2) and leading to its dissociation from mdm2, which otherwise targets p53 for degradation (66). Ser15 phosphorylation promotes interactions with CBP/p300, required for transcriptional activity (24, 48), and is involved in disrupting the inhibition by mdm2 (66). We demonstrate pX-dependent Ser15 phosphorylation (Fig. 8D), which corresponds to Ser18 in murine p53, within 1 h following the onset of pX-mediated apoptosis. Whether pX-dependent activation of p38 MAP kinase and JNK pathways mediates p53 phosphorylations is under investigation.

Neutralization of FasL and TNF-α rescues the initiation of pX-mediated apoptosis. To substantiate a role for FasL and TNF-α induction by pX signaling in pX-dependent apoptosis, neutralizing antibody for FasL or TNF-α was added to apoptotic 4pX-1 culture media alone or together (Fig. 9). FasL- or TNF-α-neutralizing antibody inhibited pX-dependent apoptosis by 30 to 50% at the 12- to 24-h interval; by 36 h, neither antibody was effective in significantly rescuing cells from pX-dependent apoptosis. Importantly, the addition of both FasL- and TNF-α-neutralizing antibodies rescued by 70% the early phase (12 to 24 h) of pX-mediated apoptosis, whereas at the later time (36 h), apoptosis was reduced by 50% (Fig. 9A). Addition of control, unrelated immunoglobulin G (2 μg/ml) to apoptotic 4pX-1 cultures did not rescue pX-dependent apoptosis (data not shown). The rescue of pX-mediated apoptosis by addition of neutralizing TNF-α antibody had no affect on the activation of the p38 MAP kinase or JNK pathways, as assessed by transient-transfection trans-reporter assays (Fig. 9B) and by directly monitoring the activation of the p38 MAP kinase and JNK enzymes (Fig. 9C). Accordingly, these observations (Fig. 9) substantiate both the mechanism and role of FasL and TNF-α induction by pX in initiating pX-mediated apoptosis.

DISCUSSION

Combined signaling of p38 MAP kinase and JNK pathways in pX-mediated apoptosis. We have identified the signaling of both the p38 MAP kinase and JNK pathways as an essential

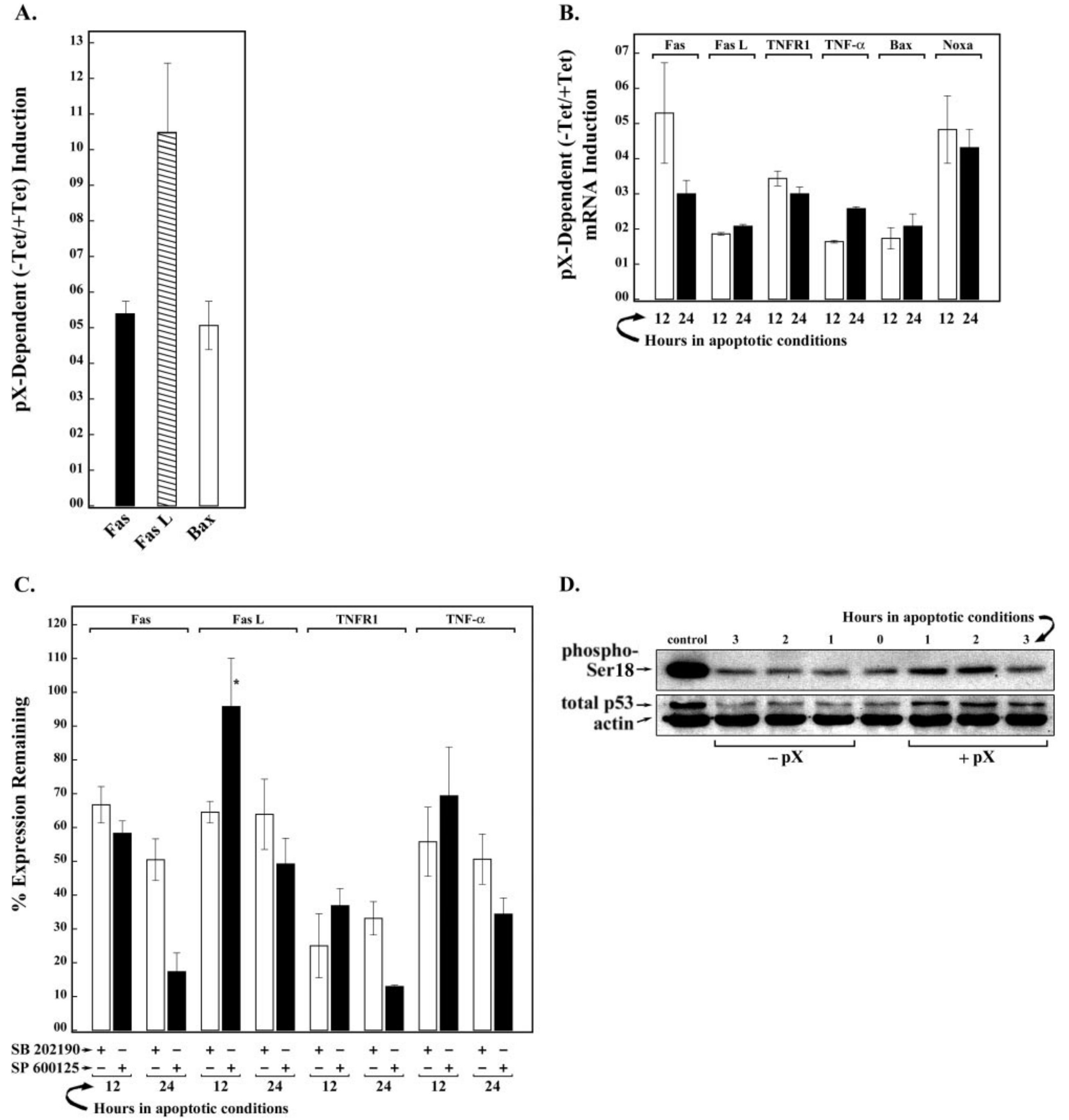
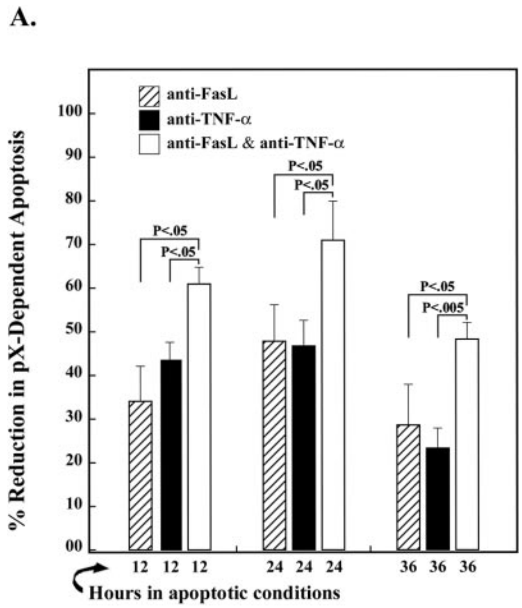
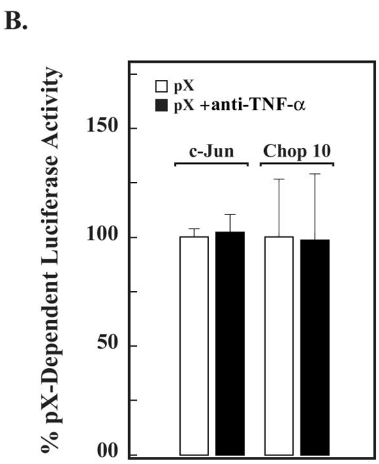


FIG. 8. Transcriptional induction of Fas/FasL and TNFR1/TNF-α in pX-mediated apoptosis. (A) Transient transfections of Fas-luciferase (30 ng), FasL-luciferase (100 ng), and Bax-luciferase (30 ng) in apoptotic 4pX-1 cultures. Plasmid DNAs added to 4pX-1 cultures 8 h prior to day 0 were grown in the presence (+Tet) or absence (−Tet) of pX and harvested on day 2. Results are from three independent assays, performed in triplicate, and quantities are per microgram of protein extract. (B) Real-time PCR of RNA isolated from 4pX-1 cells grown in the presence or absence of pX for 12 and 24 h following the onset of apoptosis. pX-dependent induction was calculated relative to that of 18S RNA. (C) Real-time PCR monitoring of Fas/FasL and TNFR1/TNF-α mRNA expression in apoptotic 4pX-1 cultures with (+) or without (−) SB 202190 (5 μM) or SP 600125 (5 μM). Results in panels B and C represent data from three independent RNA preparations for each PCR that were analyzed by using identical triplicate experiments ($P < 0.05$). * indicates the absence of statistical significance. (D) Western blot analysis of p53 and phospho-p53 (Ser18 of murine p53 or Ser15 of human p53) with whole-cell extracts (20 μg) isolated at the indicated intervals following the onset of pX-mediated apoptosis. Results from a representative assay of three independent whole-cell extract preparations are shown.





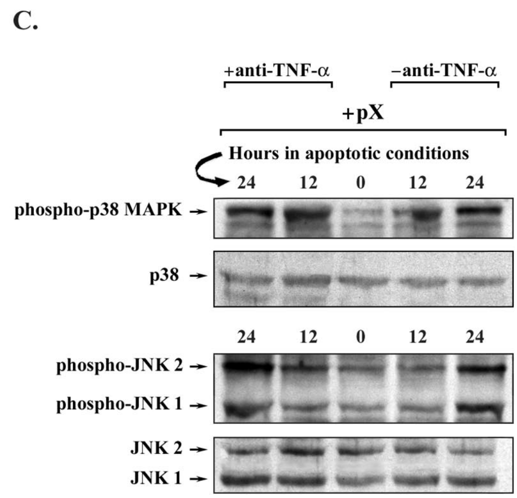


FIG. 9. Neutralization of FasL and TNF-α rescues the initiation of pX-mediated apoptosis. FasL antibody (1 μg/ml) and/or TNF-α antibody (1 μg/ml) was added as indicated for 12 to 36 h to apoptotic 4pX-1 cultures. Quantification of radioactive DNA fragmentation assays was with ImageQuant, version 5.0 (Molecular Dynamics). Results are representative of three independent experiments. (B) Transient trans-reporter assays of apoptotic 4pX-1 cultures grown in the presence or absence of TNF-α antibody (1 μg/ml); pFR-luciferase (100 ng) was cotransfected with c-Jun-Gal4 (30 ng) or pFR-luciferase (30 ng) and Chop-10-Gal4 (30 ng) as a function of pX expression. Results are representative of three independent assays performed in triplicate. (C) Western blot analyses of phospho-p38 MAP kinase and phospho-JNK in cellular extracts isolated at the indicated times following the onset of pX-dependent apoptosis and grown in the presence (+) or absence (−) of TNF-α antibody (1 μg/ml). Results of an assay representative of three independent experiments are shown.

component in pX-mediated apoptosis. pX expression in less differentiated hepatocytes mediates the sustained activation of both the JNK (73) and p38 MAP kinase (72) pathways and sensitizes cells to apoptosis (69). Although accumulated evidence implicates the p38 MAP kinase and JNK pathways in apoptosis (85), the experimental models studied, e.g., overexpression of constitutively active ASK-1, JNK1, and MEKK1 (31, 39, 41, 51) or the use of potent apoptotic stimuli (8, 56, 67, 79), have favored the identification of only the JNK pathway in apoptosis. Strong apoptotic stimuli likely override and/or mask the participation of the p38 MAP kinase pathway in favor of the JNK pathway, known to control the mitochondrial release of cytochrome *c* and the activation of caspase 9 (22, 50).

In the present study, we employed a tetracycline-controlled, pX-expressing cellular model and investigated the early signaling events involved in pX-mediated apoptosis. The significance of our conditional model is that (i) the expression level of pX (49, 71, 73) resembles physiologically relevant pX expression levels as they occur during viral infection (18) and (ii) pX is weakly apoptotic since it sensitizes hepatocytes to apoptosis only in response to weak apoptotic stimuli (43, 69). Accordingly, our cellular model of apoptosis contrasts with those of studies employing strong apoptotic inducers (57) and provides information likely to resemble, in vivo, physiologically relevant apoptotic stimuli. In agreement with similar observations by others (43, 69), we show that pX sensitizes 4pX-1 cells to apoptosis (Fig. 1A).

Importantly, we provide the first evidence of the mechanism involving the signaling of both the p38 MAP kinase and JNK pathways in mediating apoptosis (Fig. 2, 4, 5, and 8) in response to a weak apoptotic stimulus. The p38 MAP kinase inhibitor SB 202190 rescues by 80% the initial phases of pX-dependent apoptosis (Fig. 2D), whereas at later times (48 h) the contributions of both the p38 MAP kinase and JNK pathways to pX-dependent apoptosis are equal (Fig. 2D). This result demonstrates that the p38 MAP kinase pathway plays a major role in the initiation of pX-mediated apoptosis and agrees with the idea of an early, pX-dependent activation of

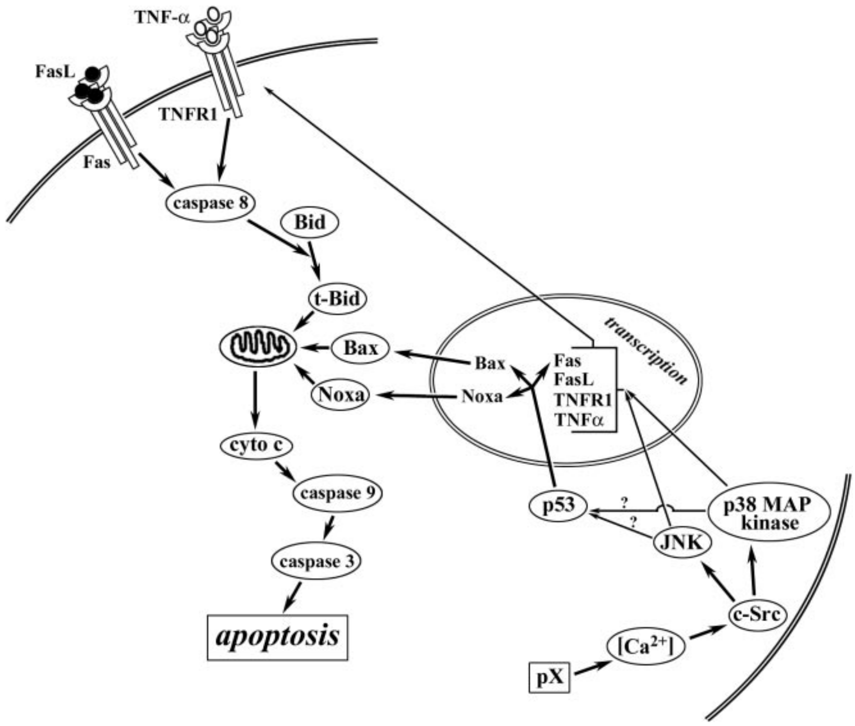


FIG. 10. Diagram of the mechanism of pX-mediated apoptosis. pX expression under conditions of growth factor deprivation induces sustained activation of the p38 MAP kinase and JNK pathways, leading to transcriptional induction of Fas/FasL, TNRF1/TNF-α, and the p53-regulated Bax and Noxa genes. pX-dependent expression of death receptors and their corresponding ligands induces caspase 8 activation and Bid cleavage. t-Bid, together with pX-induced Bax and Noxa, promotes cytochrome *c* release and caspase 9 activation, leading to apoptosis.

p38 MAP kinase versus the delayed activation of JNK (Fig. 2B).

The significance of this early and major involvement of the p38 MAP kinase pathway in apoptosis in response to weak apoptotic signals is as follows: (i) the p38 MAP kinase pathway both induces the expression of death receptor family genes (Fig. 8) and activates the G_2/M checkpoint by inhibiting Cdc2 kinase (Fig. 7), thus positioning the cell towards apoptosis, and (ii) the JNK pathway, in contrast to the p38 MAP kinase pathway, is known to activate NF-κB (54), which has antiapoptotic effects (70). Accordingly, we propose that the early and major participation of the p38 MAP kinase pathway in apoptosis is important in the initiation of apoptosis in vivo when weak, physiologically relevant apoptotic stimuli are involved. In support of this proposal, it is well accepted that suppression of apoptosis is an important aspect of tumor development (27). Interestingly, recent studies demonstrated that the activity of the p38 MAP kinase pathway is reduced in human liver tumor samples compared with that in their corresponding nontumorous controls (38) and that the loss of p38 MAP kinase activation is associated with increased tumorigenesis in mutant cells (10).

Caspase 8 is the initiator of pX-mediated apoptosis. Our observations identify caspase 8 as the initiator caspase in pX-dependent apoptosis (Fig. 3 to 5). Specifically, the use of cell-permeable caspase inhibitors (Fig. 3) demonstrated that the inhibition of caspase 8 reduces apoptosis by approximately 60% in the 12- to 36-h interval but that inhibition of caspase 9 blocks approximately 20 to 40% of pX-dependent apoptosis in the same interval. Western blot analyses (Fig. 4) and in situ detection of activated caspase 8 and activated caspase 9 (Fig. 5) further confirm the early activation of caspase 8, followed by the delayed activation of caspase 9. Additional evidence in support of this conclusion is derived from the kinetics of Bid cleavage (Fig. 6), a known substrate of caspase 8 (53, 55). Bid is a proapoptotic Bcl-2 family member, critical in hepatocyte apoptosis induced by Fas/TNFR1 engagement (80, 86). We observe that (i) Bid cleavage starts by 12 h following the onset of pX-dependent apoptosis, in agreement with the early (Fig. 4 and 5) and major (Fig. 3) activation of caspase 8, and (ii) Bid cleavage is significantly inhibited by the addition of the p38 MAP kinase inhibitor SB 202190 and to a smaller extent by SP 600125 (Fig. 6A), observations that agree with the early and major contribution of the p38 MAP kinase pathway in pX-mediated apoptosis (Fig. 2).

Furthermore, activated Bid translocates to mitochondria and induces cytochrome *c* release, thus mediating the activation of caspase 9 (86). Interestingly, cytosolic cytochrome *c* is detected starting at 12 h, in parallel with Bid activation (Fig. 6). Likewise, accumulation of the maximum amount of cytosolic cytochrome *c* parallels the appearance of active caspase 9, shown either by Western blotting (Fig. 4B) or by the direct, in situ detection of caspase 9 (Fig. 5C and D). We conclude that the activation of caspase 8, mediated by the pX-dependent activation of p38 MAP kinase and JNK pathways, mediates Bid activation, leading to cytochrome *c* release and activation of caspase 9. Importantly, Bid null mice (87) have demonstrated the importance of Bid activation in Fas-mediated apoptosis in the liver, thus supporting our findings.

pX-dependent transcriptional induction of proapoptotic genes. The mechanism of pX-dependent apoptosis, mediated by the activation of both the p38 MAP kinase and JNK pathways, involves the transcriptional induction of two classes of proapoptotic genes: those activating the death receptor, extrinsic apoptotic pathway and those involving the mitochondrial, intrinsic apoptotic pathway.

We show with apoptotic 4pX-1 cultures the pX-dependent transcriptional induction of the Fas/FasL and TNFR1/TNF-α genes (Fig. 8) that activate the death receptor pathway (61). Importantly, the addition of both FasL- and TNF-α-neutralizing antibodies reduces by 70% the initiation of pX-dependent apoptosis (Fig. 9A). This evidence directly substantiates the idea that FasL and TNF-α induction by pX initiates pX-mediated apoptosis. Furthermore, the addition of TNF-α-neutralizing antibody did not decrease the level of activation of the p38 MAP kinase and JNK pathways (Fig. 9B and C), thus supporting the significance of the pX-dependent activation of these pathways in pX-mediated apoptosis.

In addition to the pX-dependent induction of the death receptor genes in apoptotic 4pX-1 cultures, pX also induces the expression of the p53-regulated Bax and Noxa genes (Fig. 8B) involved in activation of the intrinsic apoptotic pathway. Importantly, the pX-dependent transcriptional induction of both classes of genes occurs via the activation by pX of both the p38 MAP kinase and JNK pathways (Fig. 8C). This result agrees with those of earlier studies by others investigating the mechanism of their transcriptional regulation (28, 35, 52, 59, 89). In addition, the role of the p38 MAP kinase and JNK pathways in transcription of the p53-regulated Bax and Noxa genes (data not shown) likely involves p53 phosphorylation (12, 36). The increased pX-dependent p53 protein levels and the increased pX-dependent Ser18 phosphorylation (Fig. 8D), detected at 1 h following the onset of pX-mediated apoptosis, agree with the pX-dependent transcriptional induction of Bax and Noxa (Fig. 8B). More-extensive analyses of pX-dependent modifications of p53 are in progress (W.-H. Wang and O. M. Andrisani, unpublished observations) to link p38 MAP kinase and JNK activity to p53 function in pX-mediated apoptosis.

In summary (Fig. 10), we demonstrate that pX-mediated apoptosis involves the sustained activation by pX of both the p38 MAP kinase and JNK pathways, resulting in the transcriptional induction of the Fas/FasL and TNFR1/TNF-α genes. In turn, Fas/FasL and TNFR1/TNF-α expression activates the death receptor pathway and caspase 8, followed by the involvement of the intrinsic mitochondrial apoptotic pathway, which leads to the release of cytochrome *c* and the activation of caspase 9.

Our observations agree with (i) the results of transgenic studies of the role of pX in abolishing Bcl-2 protection against Fas apoptosis in liver (74), (ii) the idea that pX has a role in apoptosis via inhibition of the caspase 8 inhibitor c-FLIP (43), and (iii) clinical data of the role of Fas and caspase 8 activation in liver disease (32). Specifically, up-regulation of Fas expression occurs in the periportal liver region of chronic HBV and HCV patients and in patients with HBV-related cirrhosis (32). This region is occupied by slightly differentiated (4pX-1-like) hepatocytes (72). In addition, transgenic mouse studies revealed that Fas-mediated apoptosis plays a crucial role in the cytotoxicity of HBV-specific cytotoxic T lymphocytes (CTLs) against virus-infected hepatocytes (1, 45). Fas-mediated apoptosis plays a critical role in regulating the immune system and tissue homeostasis and is implicated in diseases involving the pancreas and thyroid (14, 30). We propose that the apoptosis mechanism we describe herein is a general mechanism that is not limited to the liver.

Regarding the implications of our study for the pathophysiology of HBV infection, the pX-dependent transcriptional induction of FasL/TNF-α in apoptotic 4pX-1 cell is intriguing. What is the advantage to noncytopathic HBV in killing the hepatocyte that sustains its replication? It is important to consider that both the p38 MAP kinase and JNK pathways are crucial to liver development, growth, and homeostasis (3, 26, 83). Accordingly, the strength of the signal initiated by pX expression in mediating the activation of these mitogenic and stress pathways, in conjunction with other hepatocyte microenvironmental signals (TNF-α, TGF-β, and hepatocyte growth factor), may favor either growth or apoptosis. Interestingly, FasL mRNA and protein have been detected in hepatocellular carcinoma tissues (68), suggesting that its expression is linked to hepatocarcinogenesis. It is also interesting to consider the possibility that during chronic HBV infection, FasL and TNF-α expression induced by pX in an infected hepatocyte may result either in hepatocyte apoptosis or in the apoptosis of virus-specific CTLs (4, 84). The latter possibility provides a mechanism for the infected hepatocyte to evade immune attack and thus contribute to chronicity and liver cancer development (60). In support of this concept, it has been demonstrated that chronic HBV patients have a much less vigorous CTL response than that of patients with acute HBV infection (16) and that FasL induction in HepG2 cells mediates lymphocyte killing in vitro (68).

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